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1a. REPORT SECURITY CLASSIFICATION	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S): AFOSR-TR- 90 0940
6a. NAME OF PERFORMING ORGANIZATION: Department of Biol.Sci. Miss. State University	6b. OFFICE SYMBOL (If applicable)
6c. ADDRESS (City, State, and ZIP Code): P.O. Box 6156 Miss. State, MS 39762	7a. NAME OF MONITORING ORGANIZATION: AFOSR
	7b. ADDRESS (City, State, and ZIP Code): Bld 410 Bolling AFB, DC 20332
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: AFOSR	8b. OFFICE SYMBOL (If applicable): NL
8c. ADDRESS (City, State, and ZIP Code): AFOSR/NL Building 410 Bolling AFB, DC 20332-6448	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: AFOSR-89-0361
	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 61102F; PROJECT NO. 2312; TASK NO. A5; WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification)

Relationship of Selected Functions of Activated Macrophages

12. PERSONAL AUTHOR(S)

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13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 89/6/15 TO 90/6/14	10 August 1990	43

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
			Macrophage, Nitric oxide, Nitrite, Nitrate Tumor cytolysis, Listeria monocytogenes, Immunotoxicology,

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The objective of this project was to examine relationships of selected metabolic capabilities and key immunological functions of activated macrophages. Consistent association or a single example of dissociation between two parameters would indicate or eliminate the possibility of common induction pathways or functional interdependence of these parameters. The results indicate that thiol production, capacity to produce H_2O_2, and tumor cytotoxicity are often induced by the same stimuli, but in one case, H_2O_2 production was not affected by stimuli which increased the other two parameters. The commonly accepted idea that highly activated (tumoricidal) macrophages are poor antigen processing and presenting cells was confirmed, but some tumoricidal activity was noted in macrophages which were excellent antigen processing and presenting cells. The ability of macrophages to kill *Listeria monocytogenes* did not correlate well with ~~any of the~~ other metabolic capabilities or immunological functions examined. Using culture medium without L-arginine (which decreased the production of nitrogen oxides) decreased anti-*Listeria* activity of macrophages, in some, but not all cases. (continued on reverse side)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	
22a. NAME OF RESPONSIBLE INDIVIDUAL: T. Jan Cooney, Lt Col USAF	22b. TELEPHONE (Include Area Code): 202 767-5021
	22c. OFFICE SYMBOL: NL

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This indicates that the importance of this recently recognized anti-microbial mechanism depends on a number of factors, including the microbe used, the mouse strain, and the activation status of the macrophages. Experiments done to assess the possible role of nitrogen oxides in anti-microbial effects of human neutrophils suggested that these cells cannot produce enough nitrogen oxides to contribute to their anti-microbial activity. An new assay for neutrophil viability and respiratory burst activity was developed to confirm that the human neutrophils used in these studies were viable and metabolically active.

RELATIONSHIPS OF SELECTED FUNTIONS OF ACTIVATED MACROPHAGES

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10 August 1990

Final Report for Period 15 June 1989-14 June 1990.

Prepared for

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Building 410
Bolling AFB
Washington DC 20322-6448

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a. Objective

The objective of this project was to examine the relationships between several metabolic capabilities acquired by activated macrophages (destructive catabolism of antigen, generation of reactive oxygen intermediates after appropriate stimulation, and generation of thiols) and the key biological functions of macrophages (anti-tumor and anti-microbial activity and processing and presenting antigen to initiate specific immune responses).

The project was conducted essentially as proposed, except that reactive nitrogen intermediates (as indicated by the presence of nitrite ions in the culture medium) were examined instead of destructive catabolism of antigen or reactive oxygen intermediates as a possible modulator of key biological functions. This change was based on several reports indicating the importance of nitrogen oxides in the cytostatic or cytotoxic action of macrophages on some tumor cells and microbes (1,2,3). In addition, production of nitrates and nitrites by primed or activated macrophages does not require a respiratory burst-triggering stimulus (4), and is more likely to occur concomitantly with key biological functions than those metabolic activities which require triggering. The only other deviation from the initial proposal was the use of multiple mouse strains (C3H/HeN, DBA/2J, Balb/c, and CD-1) instead of multiple eliciting agents (*Salmonella typhimurium* and lipopolysaccharide) to generate macrophages with various metabolic and biological capabilities.

Additional experiments not described in the original proposal were done to assess the relevance of nitrites and nitrates as biological mediators produced by human neutrophils. Substantial nitrite production by inflammatory neutrophils from rats has been reported, and it has been suggested that nitrates may be an important mediator of anti-microbial activity and/or tissue damage (5). A novel assay for neutrophil viability and respiratory burst activity was developed, and nitrite production by neutrophils from several donors was examined in response to several activating and triggering agents.

b. Status of Research Effort

1. Experimental Design and Procedures

Mice- All mice were females and were obtained from Charles

River Labs. According to the supplier, the mice were free of antibodies to common mouse viruses. Animals were 6-12 weeks of age when used in experiments.

Isolation and Culture of Macrophages- For isolation of proteose peptone-elicited macrophages, 1.5ml of sterile proteose peptone (Difco Labs) broth was injected intraperitoneally. Three days later, macrophages were obtained by peritoneal gavage with 8ml of ice cold RPMI 1640. Macrophages from 10-25 mice were pooled, washed twice and resuspended in RPMI 1640 which contained 5% fetal bovine serum and penicillin (100U/ml)/streptomycin (0.1mg/ml)(complete medium). To assess anti-bacterial activity in the absence of L-arginine, some cultures were washed with L-arginine-free RPMI 1640 (Selectamine Kit, Gibco Biological) and incubated for 1 hour in this medium prior to anti-bacterial assays. The anti-bacterial assays were then performed in RPMI 1640 with or without L-arginine and with 5% fetal bovine serum. Isolation of resident peritoneal macrophages was done in the same way, but mice were not pre-treated with proteose peptone. Cell number was determined by hemocytometer, and differential staining (Camco Quick Stain II, Baxter Scientific) was used to determine the percentage of macrophages in the cell suspension. This value varied among the strains used and between resident and elicited cells, but was in the range of 60-90% in all experiments. Macrophages were allowed to adhere to the wells of microtiter plates for 2 hours at 37C, and other cells were removed by two washes with complete medium. The number of macrophages added per well was determined on the basis of the total cell density and the percentage of macrophages in the suspension. The fetal bovine serum and culture medium used in this study contained less than 0.09ng/ml of endotoxin. As noted in subsequent sections, increased thiol production by macrophages in response to activation stimuli is a very consistent phenomenon. In two experiments, the thiol production by unactivated macrophages was higher than normal and was not increased further by activation stimuli. This was taken as an indication of an unapparent infection in the mice or endotoxin contamination in culture, and the results of those experiments were considered invalid and are not reported.

Activation of Macrophages In Culture- A lymphokine-rich supernatant fluid (LK SUP) was obtained by stimulating splenocytes ($5x10^6$/ml) from C3H/HeN mice with concanavalin A (5µg/ml) for 24 hours. A control supernatant fluid (CONT. SUP) was obtained by adding an equivalent amount of concanavalin A to untreated splenocytes at the end of the 24 hour culture and harvesting the supernatant 5 minutes later. Purified recombinant murine

interferon-gamma (IFN) was kindly provided by Genentech, Inc. Lipopolysaccharide (LPS) derived from *Escherichia coli* 011:B was purchased from Sigma Chemical Co. and was used at a final concentration of 10ng/ml in all experiments. For all procedures except the tumor cytolysis assay, macrophages were activated for 20 hours in culture with the appropriate activating agent(s) before being examined in functional assays. For the tumor cytolysis assay, macrophages were activated in culture for 4 hours before addition of radiolabeled tumor cells. The activating agent(s) remained in the culture during the 16 hour incubation for tumor cytolysis. Thus, by the end of the tumor cytolysis assay the macrophages had been exposed to activating agent(s) for approximately the same period of time as in the other assays.

Assays of Macrophage Metabolic and Immunological Activity- All assays were performed in triplicate in 96-well flat bottom microtiter plates. Thiols in macrophage culture supernates were quantitated by our modification of the DTNB method (6). Hydrogen peroxide was measured by spectrophotometric determination of the oxidation of phenol red by culture supernates (7). For these assays, macrophages were cultured at $8x10^5$/well (0.2ml/well) with activating agents for 20 hours then washed and supplied with fresh culture medium (200µl/well). Phorbol myristate acetate (PMA, 200nM, final concentration) was added to some wells to trigger the respiratory burst. Hydrogen peroxide was assayed 1 hour after addition of PMA, and thiols were assayed 3 or 4 hours after addition of PMA.

Tumor cytolysis was assessed by measuring the release of ^{51}Cr from labeled P815 tumor cells ($6x10^4$/well) 16 hours after they were added to wells containing $4x10^5$, $2x10^5$, or $1x10^5$ macrophages. The ability of macrophages to kill the intracellular pathogen *Listeria monocytogenes* (Strain EGD, American Type Culture Collection) was assessed essentially as described by Kagaya et al. (8). Briefly, bacteria (approximately 10^6/well) were added to macrophages ($3x10^5$/well) in the presence of 10% normal mouse serum. After two hours at 37C, the monolayers were washed three times with complete medium to remove extracellular bacteria. Macrophages were lysed with distilled water immediately or after an additional 5 hour incubation at 37C, and viable intracellular bacteria were quantitated by the spread plate method. In some experiments, culture supernates (100µl) were obtained from wells to which bacteria were added as well as from replicate wells without bacteria, and nitrites were quantitated using the Greiss reagent as described by Keller et al. (1).

The ability of macrophages to process and present antigen was assessed using the ovalbumin-specific, H-2^k restricted T cell hybridoma 3DO26.1 (kindly provided by Dr. P. Marrack) (9). Following exposure to activating agent(s) for 20 hours, macrophage monolayers (10^5/well) were washed and incubated for 6 hours with ovalbumin (1mg/ml in complete medium) to allow time for antigen processing and presentation. The cells were washed three times with Hank's balanced salt solution (HBSS) and fixed for 30 sec. with 0.05% glutaraldeyde in HBSS. This step was necessary to prevent lysis of the T cell hybridoma by the most highly activated macrophages (10). After removal of the glutaraldehyde solution, the macrophages were washed with a 0.1M lysine solution and 10^5 3DO26.1 cells in 200µl complete medium were added to each well. When antigen in association with the appropriate MHC protein is presented to these cells, they respond by producing Interleukin 2 (IL2). IL2 in culture supernates was measured by its ability to support the survival and growth of an IL2-dependent cell line, CTLL-2 (11).

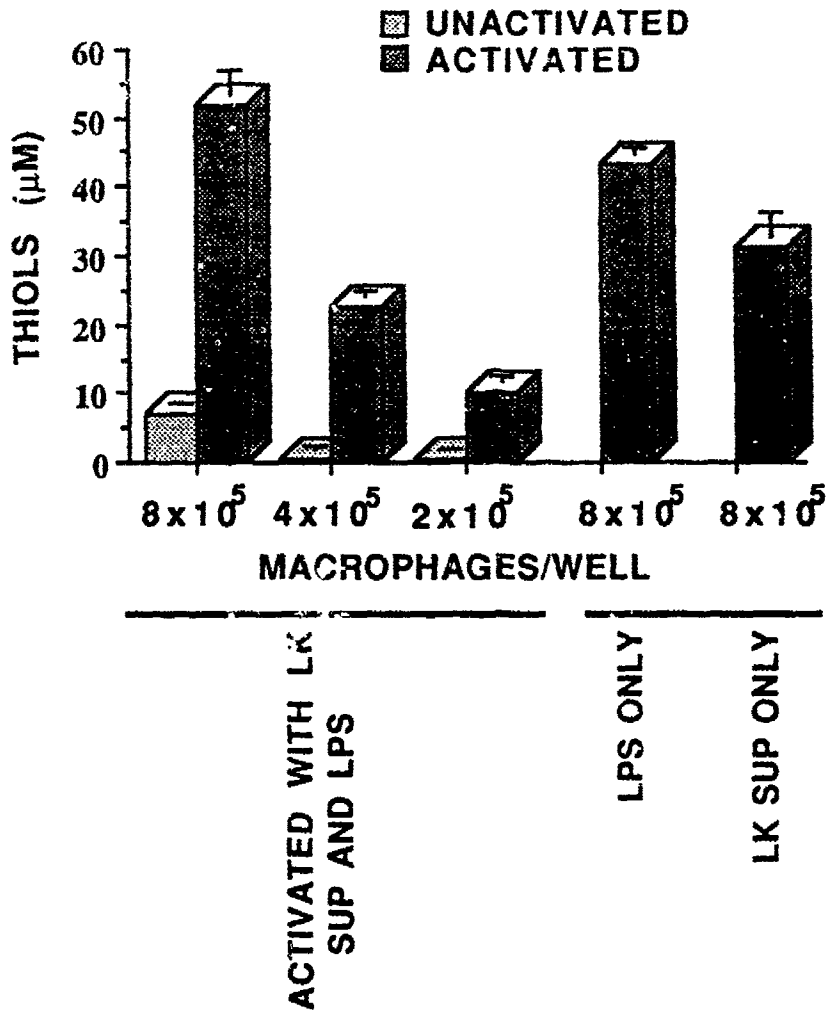
All assays were performed with duplicate or triplicate samples and results are reported as means±standard deviation. Statistical analysis (Student's t test) was performed on all data, but significant differences are generally obvious . To simplify data presentation, statistics are not reported.

2. Results and Discussion

The data in Figure 1 demonstrate the ability of a priming agent (lymphokine-rich splenocyte supernate, LK SUP), a second signal (LPS), and a combination of the two to enhance thiol production in 24 hour cultures of proteose peptone-elicited macrophages from C3H/HeN mice. This extends our findings (6) and those of two other groups of investigators that elicitation *in vivo* or treatment with LPS or tumor necrosis factor *in vitro* increases thiol production by macrophages (12, 13). The function of increased thiol production is not known, but it has been suggested that it may serve to protect macrophages and other cells in the area from the reactive oxygen intermediates produced during the respiratory burst (14) or to provide lymphocytes with a limiting nutrient (cysteine)(13). The signal transduction pathway(s) leading to enhanced cystine uptake/thiol release are not known. In this study, the relationship of the induction pathway for this response and other responses of activated macrophages were examined by assessing these responses simultaneously in several distinct populations of macrophages. In addition, consistent co-induction of functions was used as an

FIGURE 1. Production of thiols in 24 hour cultures by proteose-peptone-elicited macrophages from C3H/HeN mice. Macrophages (at the indicated number per well in 0.2 ml) were activated with a lymphokine-rich supernate of concanavalin A-stimulated splenocytes (LK SUP), with lipopolysaccharide (LPS), or with a combination. LK SUP in this and all subsequent experiments was used at a final concentration of 20% of the culture medium. LPS in this experiment and all subsequent experiments was used at a final concentration of 10ng/ml. This concentration was selected because it is suffificient to function as a second signal for the induction of tumor cytolysis, but not sufficient to induce this or other functions alone. Unactivated cultures were treated with complete medium. Activating agents remained in the cultures for the entire 24 hour culture period. The results are reported as means±standard deviation for triplicate cultures.

PRODUCTION OF THIOLS IN 24 HOUR CULTURES
BY PROTEOSE PEPTONE-ELICITED MACROPHAGES
FROM C3H/HeN MICE



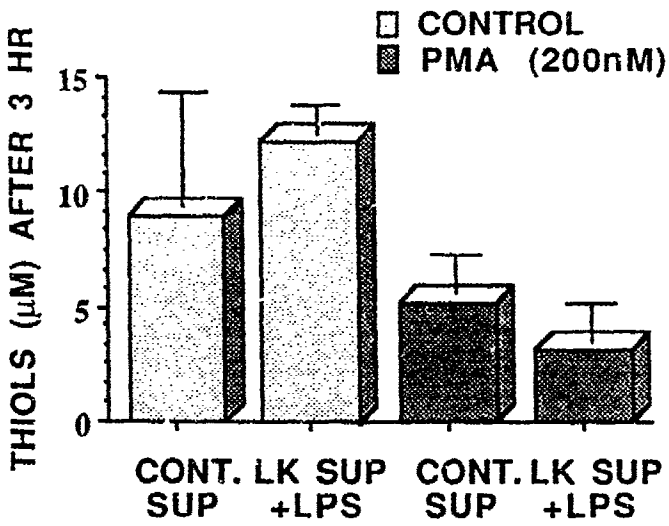
indicator of possible common biochemical pathways or mechanisms, whereas dissociation of two functions (induction of one function and failure to induce the other function) was used as a conclusive indicator of the independence of these functions.

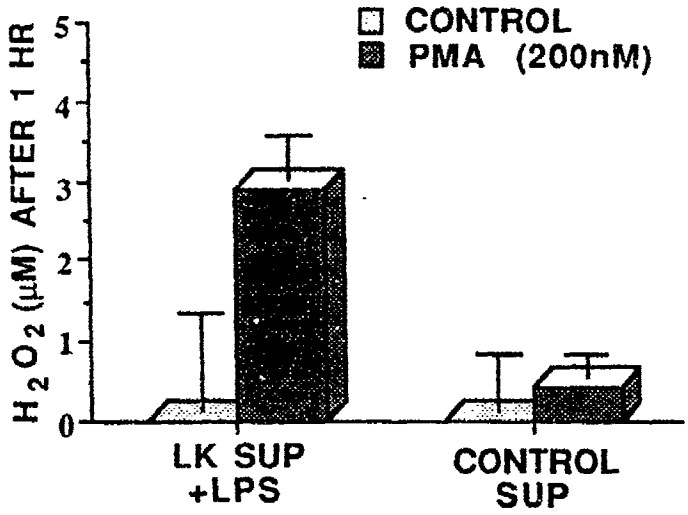
The first series of assays were conducted using proteose peptone-elicited macrophages from C3H/HeN mice. Three parameters were examined in each of two independent experiments (Figures 2 and 3). As expected, LK SUP with LPS enhanced the production of thiols and the PMA-induced production of H_2O_2. Control SUP, which contains concanavalin A, enhanced both of these parameters to a lesser extent. Thiol levels were lower in macrophage cultures in which the respiratory burst was triggered by PMA. Exogenous cysteine is oxidized more rapidly in PMA-treated cultures than in control cultures or in medium alone (results not shown). Therefore, the decreased thiol levels observed in PMA-treated cultures in this and subsequent experiments may be due to oxidation of thiols by reactive oxygen compounds produced during the respiratory burst. It is also possible that PMA-stimulated macrophages generate less thiols than non-triggered cells. Macrophages activated by LK SUP with LPS acquire the ability to lyse P815 tumor cells. In these sets of experiments, all functions tested were similarly induced by the agents used. Therefore, these experiments are consistent with the possibility that these three functions share a common signal transduction pathway.

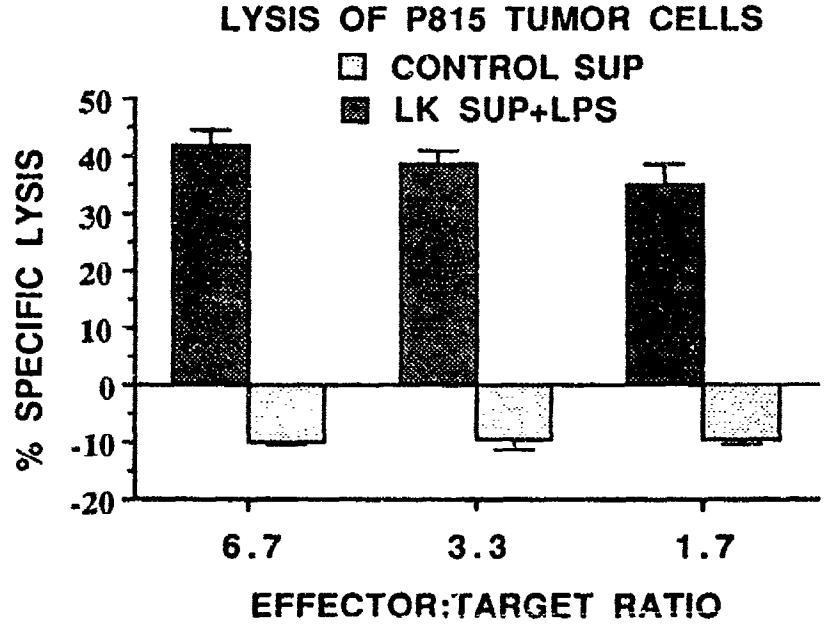
Figure 4 shows the results of two experiments which illustrate the ability of proteose peptone elicited macrophages from C3H/HeN mice to kill *Listeria monocytogenes*. Unactivated macrophages as well as macrophages exposed to LK SUP or IFN with or without LPS effectively killed the bacteria. Exposure of macrophages to LK SUP with LPS or IFN (500U/ml) with LPS increased the number of viable intracellular bacteria detected at 2 hours and 7 hours after the bacteria were added. This seems to be support the data of Campbell et al. (15) which indicate that proteose peptone-elicited macrophages lose their antibacterial activity when exposed to IFN. However, in our assay system, the number of bacteria present in the macrophages after two hours of culture reflects the number phagocytized as well as the number killed. Since extracellular bacteria are washed away at the 2 hour time point, the change in bacterial numbers from 2 to 7 hours only reflects the fate of intracellular or macrophage-bound bacteria. Therefore, the increased numbers of bacteria in cultures exposed to LK SUP with LPS or IFN with LPS may be the result of increased phagocytosis, not decreased bacterial killing. Evidence for enhancement of

FIGURES 2 and 3. Assessment of selected functions of proteose peptone-elicited macrophages from C3H/HeN mice. These are the results of two independent sets of experiments. In each experiment, thiol production (three hours after washing to remove thiols which accumulated during the activation period), H_2O_2 production in response to a triggering agent (phorbol myristate acetate, PMA), and ability to lyse tumor cells (P815 mastocytoma) were measured. The assays were done with pooled macrophages and were timed so the end points would coincide and the time of exposure to activation stimuli would be similar (20 hours). The results presented are means±standard deviation for triplicate cultures. Spontaneous release in the tumor cytolysis assay was 33.5% in Figure 2 and 20% in Figure 3.

ASSESSMENT OF SELECTED FUNCTIONS OF PROTEOSE PEPTONE-ELICITED MACROPHAGES FROM C3H/HeN MICE







ASSESSMENT OF SELECTED FUNCTIONS OF PROTEOSE PEPTONE-ELICITED MACROPHAGES FROM C3H/HeN MICE

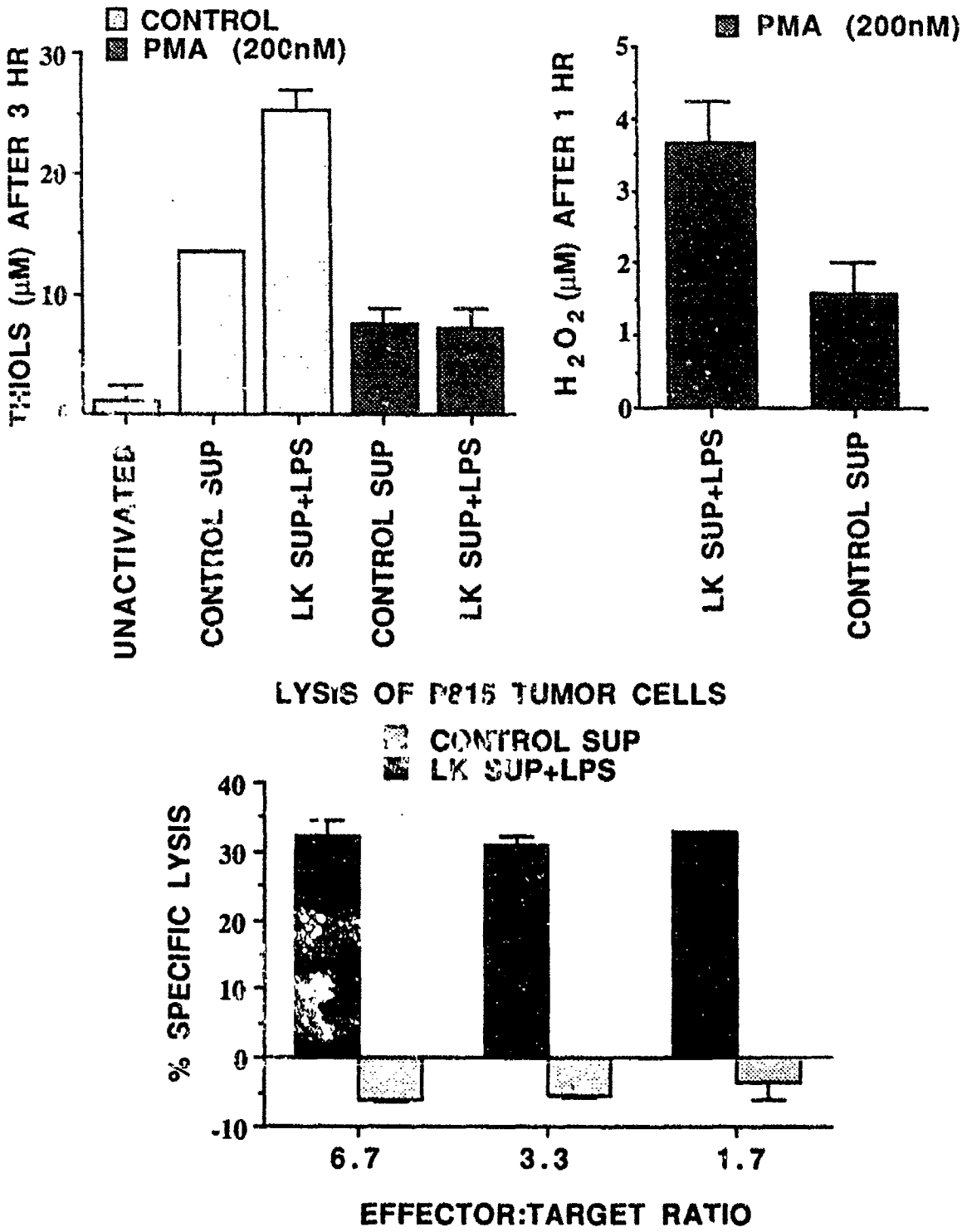
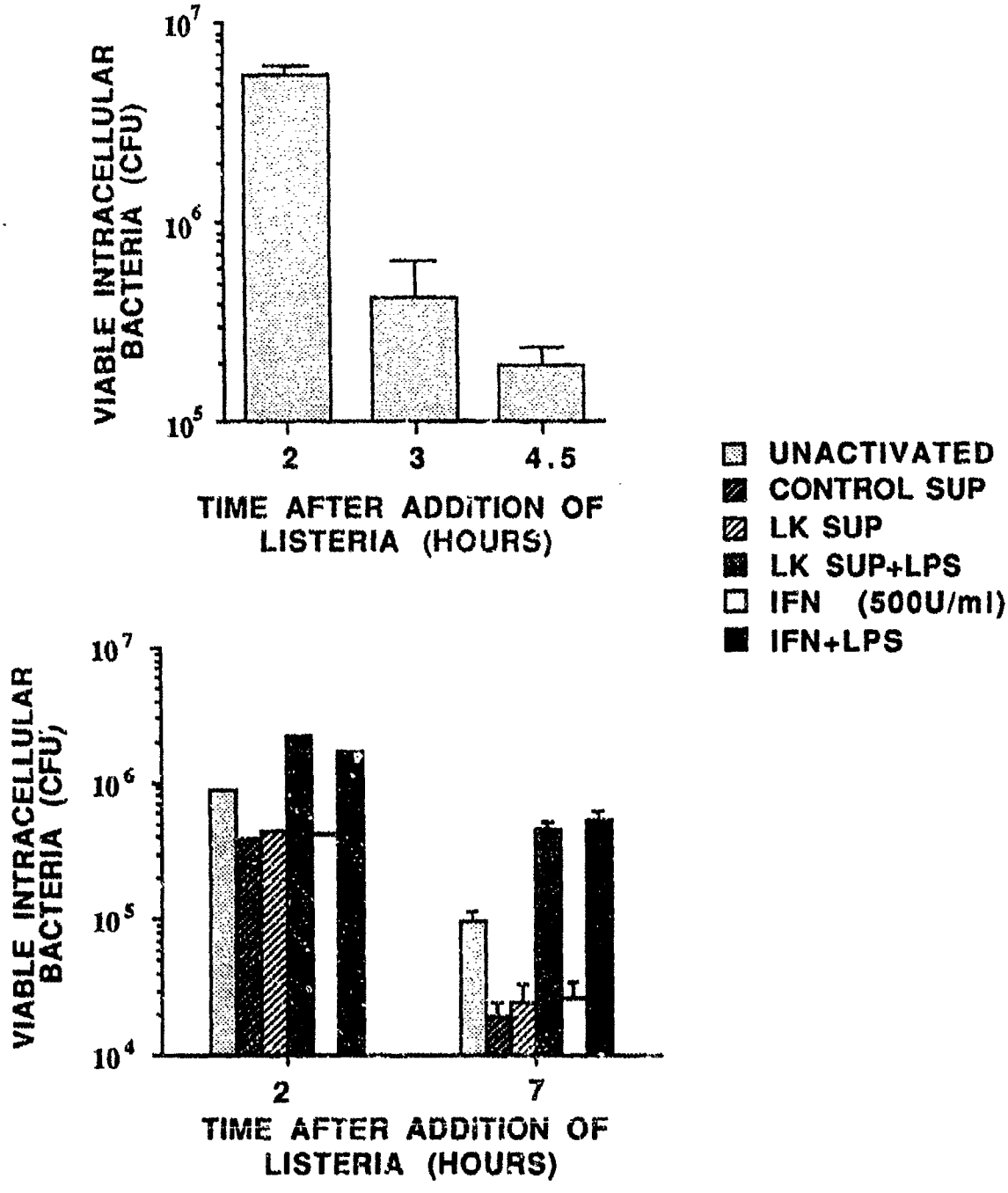


FIGURE 4 Killing of *Listeria monocytogenes* by proteose peptone-elicited macrophages from C3H/HeN mice. Results of two independent experiments are shown. In the upper panel, macrophages were allowed to adhere to microtiter plates, washed, and bacteria in 5% normal mouse serum medium were added. After 2 hours all wells were washed three times with complete medium to remove extracellular bacteria. The macrophages in some wells were immediately lysed with distilled water and the bacteria enumerated by the spread plate method. This procedure was repeated at 3 and 4.5 hours. In the lower panel, the macrophages were activated with the indicated agents for 18 hours before addtion of bacteria. Values shown are means±standard deviation for duplicate cultures, except for the 2 hour numbers in the lower panel, for which only one culture was available.

KILLING OF *LISTERIA MONOCYTOGENES*
BY PROTEOSE PEPTONE-ELICITED MACROPHAGES
FROM C3H/HeN MICE

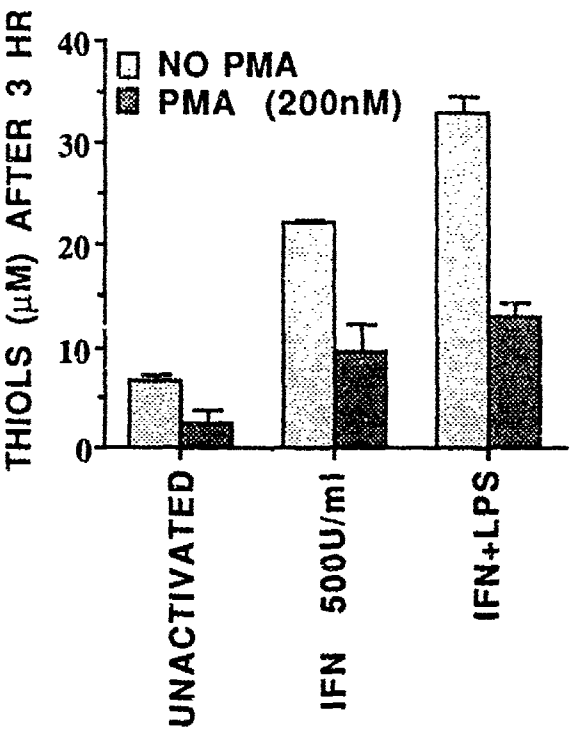


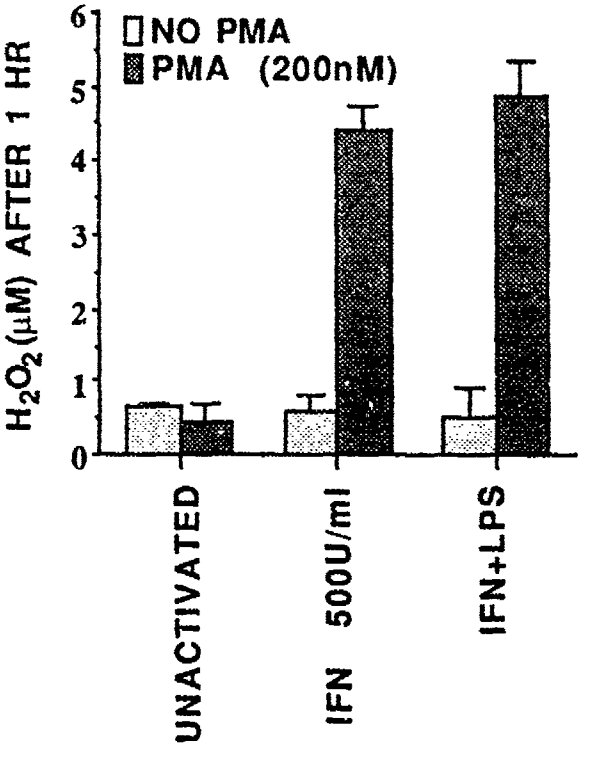
phagocytosis by LPS has been reported (16) and would be consistent with our results. Further support for this possibility can be found in Figure 12 which shows the growth or death of intracellular *Listeria monocytogenes* between 2 hours and 7 hours after addition of bacteria for all experiments in this study. This indicates only the rate of killing or growth of intracellular bacteria, not the rate of phagocytosis. For proteose peptone-elicited C3H/HeN macrophages, the rate of killing is greater in cultures containing an activating agent plus LPS than in other cultures. Therefore, the increased number of intracellular bacteria at 2 hours in cultures treated with an activating agent plus LPS is probably not due to decreased anti-bacterial activity, but to increased phagocytosis. In fact, cultures treated with LK SUP or IFN along with LPS killed bacteria at a rate 2-4 times greater than unactivated cells (Figure 12). IFN or LK sup alone slightly decreased the rate of killing compared to unactivated cultures. These results are not in agreement with those of Campbell et al. (15), who found that activation of proteose peptone-elicited macrophages for tumor cytotoxicity completely abrogated their ability to kill intracellular *Listeria monocytogenes*. This disagreement is probably due to the use of different mouse strains. Subsequent results in this study will demonstrate a remarkable interstrain variation with regard to the anti-listerial activity of unactivated and activated macrophages. The results shown in Figure 4 show that LK SUP and IFN at 500U/ml are roughly equivalent in their effects on macrophages with regard to anti-listerial activity. This is consistent with reports that the active agent in LK SUP for induction of a variety of functions is IFN-gamma (17).

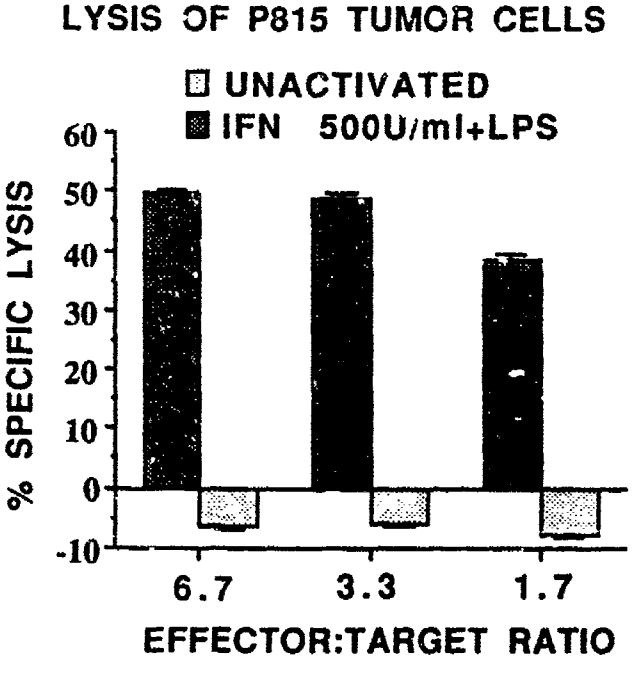
The results shown in Figure 5 demonstrate the effects of activation on 4 functional parameters of proteose peptone-elicited macrophages from DBA/2 mice. These results are very similar to those observed for macrophages from C3H/HeN mice (Figures 3 and 4). Figure 6 shows the results of a separate experiment using proteose peptone-elicited macrophages from DBA/2 mice in which antigen processing and presentation and tumor cytotoxicity were examined. Although it is generally accepted (18), there are only a few studies in which an inverse relationship between the degree of macrophage activation and ability to present antigen have been directly demonstrated (19). There are at least three possible explanations for this phenomenon. Highly activated macrophages may destructively catabolize antigen (19). The level of class II MHC protein may be down-regulated to such an extent that antigen presentation is no longer effective (20). The highly activated macrophages may become suppressive by killing or inhibiting the

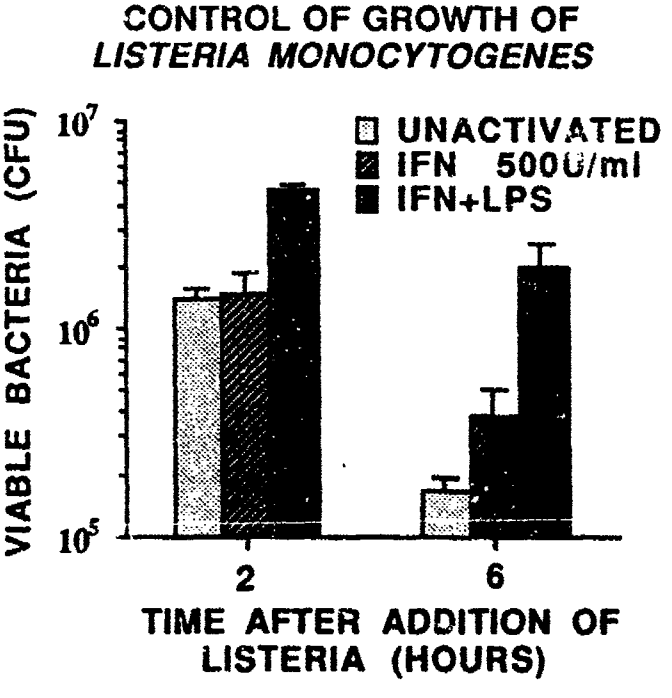
FIGURE 5. Assessment of selected functions of proteose peptone-elicited macrophages from DBA/2 mice. Pooled macrophages were placed in microtiter plates, activated with the indicated agents and tested for thiol production (three hours after washing and adding fresh medium), H_2O_2 production 1 hour after addition of PMA, lysis of P815 tumor cells and control of the growth of *Listeria monocytogenes.* Assays were timed so that all macrophages were activated for approximately the same period of time (20 hours) before the assessment of the various endpoints. Results shown are means±standard deviation for triplicate cultures. Spontaneous release of label in the tumor cytotoxicity assay was 27%.

ASSESSMENT OF SELECTED FUNCTIONS OF PROTEOSE PEPTONE-ELICITED MACROPHAGES FROM DBA/2 MICE









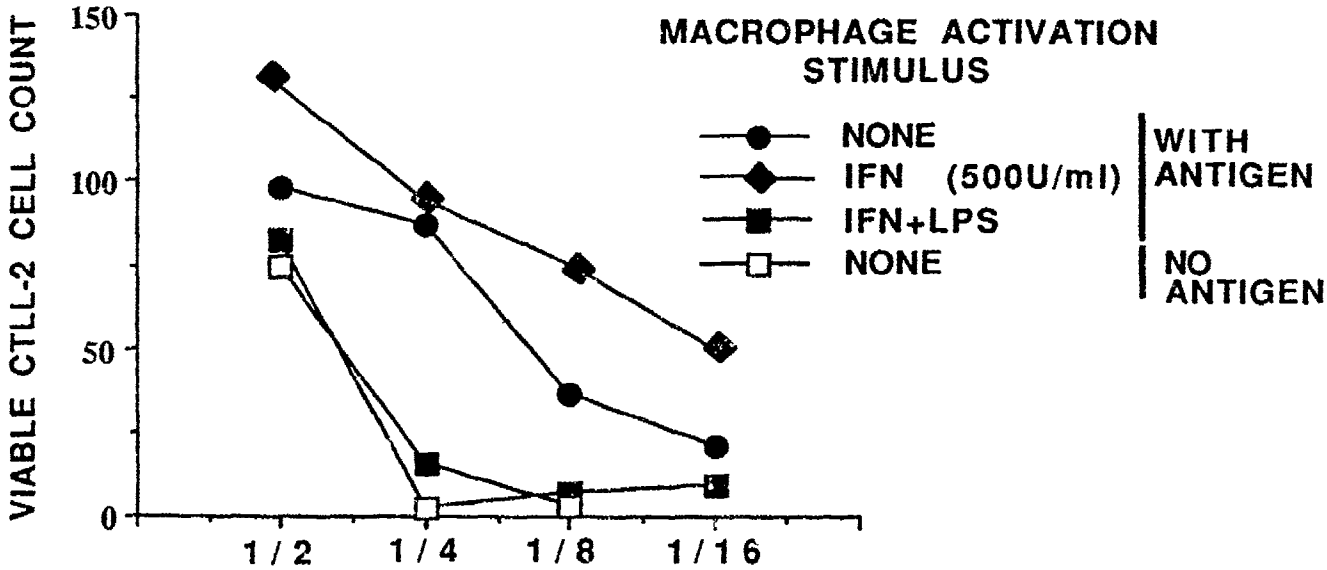
function of antigen-specific lymphocytes (21). The results presented here (Figures 6 and 7) demonstrate that antigen processing/presenting activity is essentially eliminated by exposure of macrophages to IFN plus LPS. This occurs even though the macrophages in this study were fixed with glutaraldehyde so that they could not kill or inhibit the function of the antigen specific lymphocytes. Additional studies are needed to examine the other possible mechanisms for this effect. It is interesting that IFN alone induces tumoricidal activity, whereas LPS must be present as well to decrease antigen processing/presentation. This suggests that tumor cytolytic activity and diminished antigen processing/activity may result from the activation of distinct molecular systems. Thus, the proteases implicated in tumor cytolysis may not be involved in destructive catabolism of antigen and consequent decreases in antigen processing/presenting activity. The simultaneous examination of these two parameters in the same experimental system has not been previously reported, and this system should be useful in future studies of the mechanisms involved in these functions.

The results shown in Figure 8 demonstrate that proteose peptone-elicited macrophages from Balb/c mice are different in several respects from those of C3H/HeN and DBA/2 mice. Tumor cytolysis was evident only at the higher effector:target ratios, growth of *Listeria monocytogenes* was not prevented by unactivated or activated macrophages, and unactivated macrophages produced levels of H_2O_2 comparable to those produced by activated macrophages. Thiol production and its response to stimuli was similar to that for the other strains examined. These results support previous indications that tumor cytolysis, bactericidal activity and H_2O_2 production do not share common pathways or mechanisms, even though they may be induced by the same stimuli (15, 22, 23). In particular, it is evident that the capacity to produce H_2O_2, and the ability to lyse tumor cells do not confer an ability to kill *Listeria monocytogenes*. The enhancement of thiol production and tumor cytotoxicity by activating agents indicates that the Balb/c macrophages and the activating agents were functional. Therefore, the failure of these agents to induce anti-bacterial activity demonstrates a selective deficiency of this function in this mouse strain. This is consistent with the fact that Balb/c mice are highly susceptible to lethal infection with *Listeria monocytogenes*, whereas the other strains used in this study are more resistant (24).

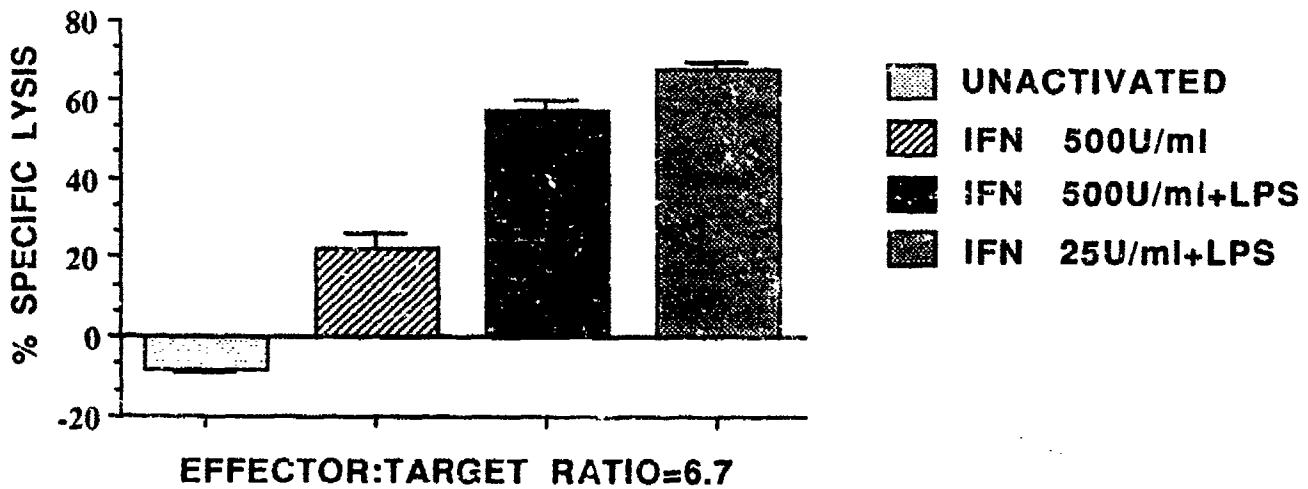
Recent evidence demonstrates a causal relationship between the production of nitric oxide and its stable products, nitrite and

FIGURES 6 and 7. Assessment of antigen processing and presenting activity and tumor cytotoxicity of proteose peptone-elicited macrophages from DBA/2 mice. In Figure 6, a pooled population of macrophages was used for both assays. Antigen processing and presentation was assessed by exposing activated (or control, unactivated) macrophages to antigen (ovalbumin) for 6 hours to allow time for processing and presentation. The macrophages were then fixed with glutaraldehyde and ovalbumin-specific, H-2 restricted T cell hybridoma cells (3DO26.1) were added to the wells. Appropriate antigen presentation results in the production of Interleukin 2 by these cells. The interleukin 2 in macrophage-T helper hybridoma supernates was measured by its ability to support survival and growth of CTLL-2 cells which are IL2-dependent. The assays were timed so that the end points were measured after approximately the same period of macrophage activation (20 hours). Figure 7 shows the results of an independent antigen processing/presentation assay. Values shown are means±standard deviation for triplicate samples.

ANTIGEN PROCESSING/PRESENTING ACTIVITY
OF MACROPHAGES FROM DBA/2 MICE



LYSIS OF P815 TUMOR CELLS BY PROTEOSE PEPTONE-
ELITICITED MACROPHAGES FROM DBA/2 MICE



ANTIGEN PROCESSING AND PRESENTATION BY PROTEOSE PEPTONE-ELICITED MACROPHAGES FROM DBA/2 MICE

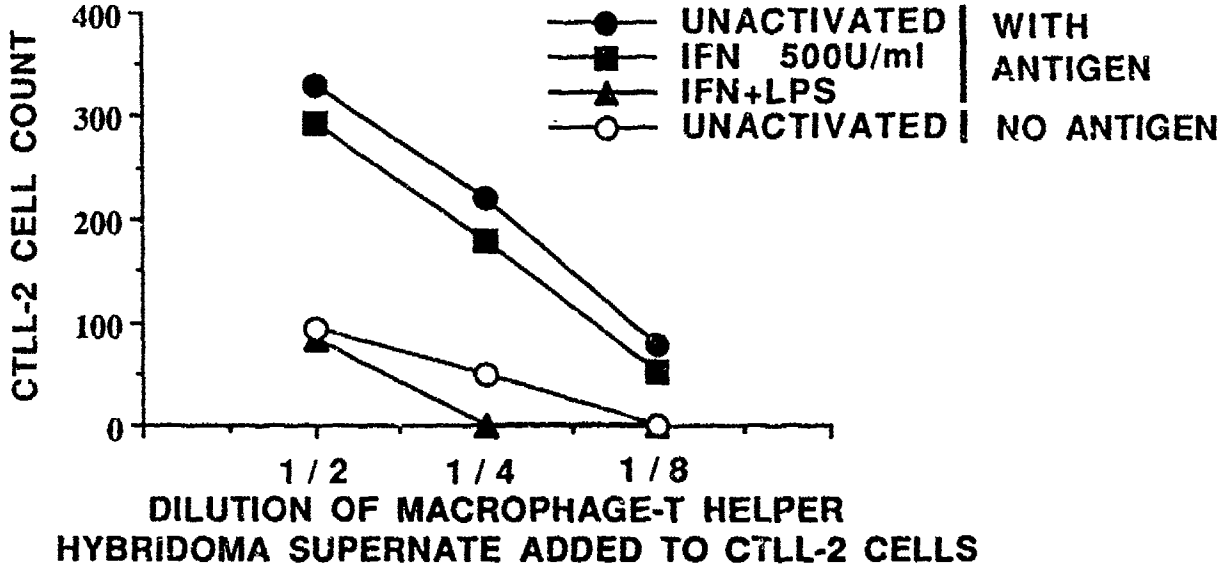
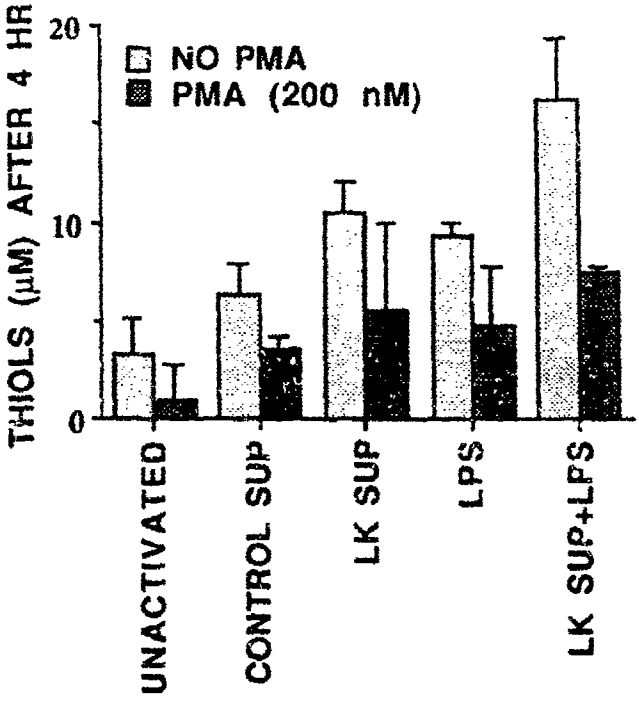
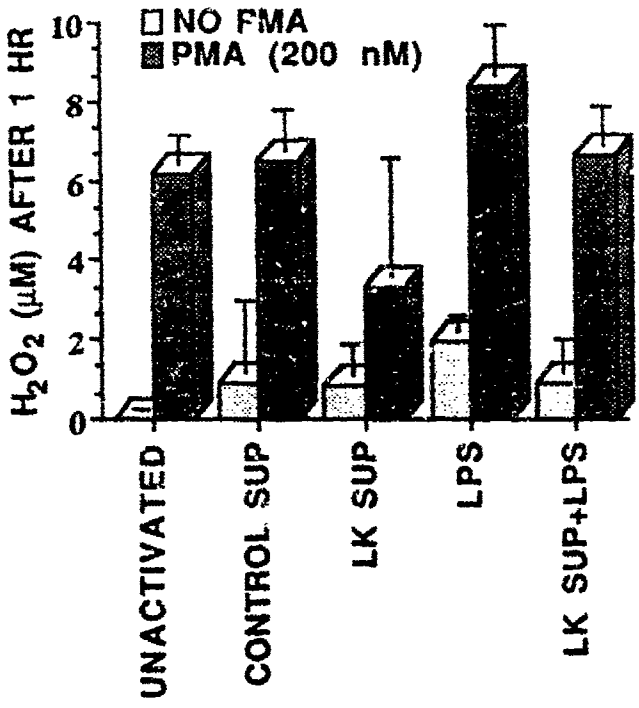


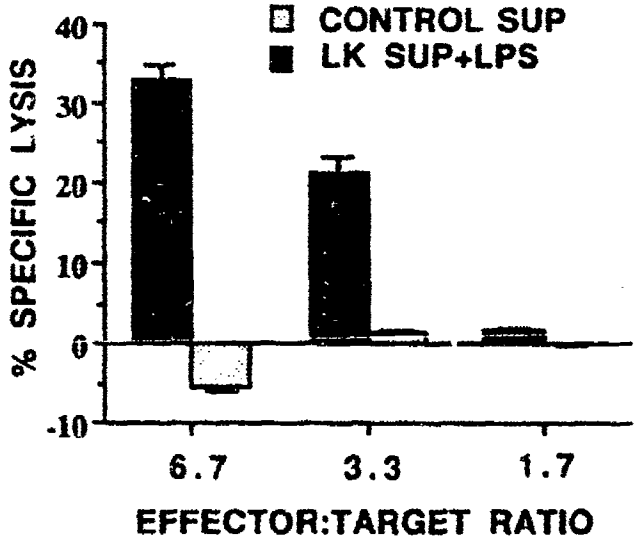
FIGURE 8. Assessment of selected functions of proteose peptone-elicited macrophages from Balb/c mice. All assays except control of *Listeria monocytogenes* growth were performed with a pooled macrophage population. The anti-*Listeria* assay was performed within one week of the other assay using mice from the same group used for the other assays. The assays were timed so that the measurements were made at approximately the same time after addition of activating agents (20 hours). Values shown are means±standard deviation for triplicate samples. Spontaneous release of label in the tumor cytotoxicity assay was 30.2%.

ASSESSMENT OF SELECTED FUNCTIONS OF PROTEOSE PEPTONE-ELICITED MACROPHAGES FROM BALB/c MICE

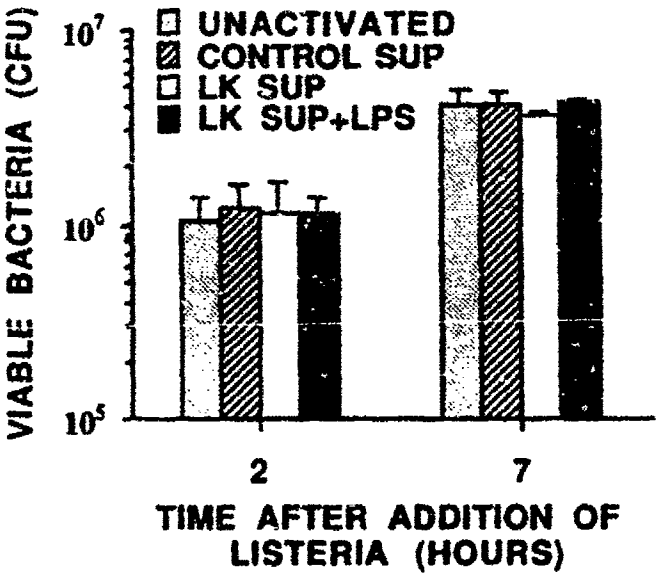




LYSIS OF P815 TUMOR CELLS



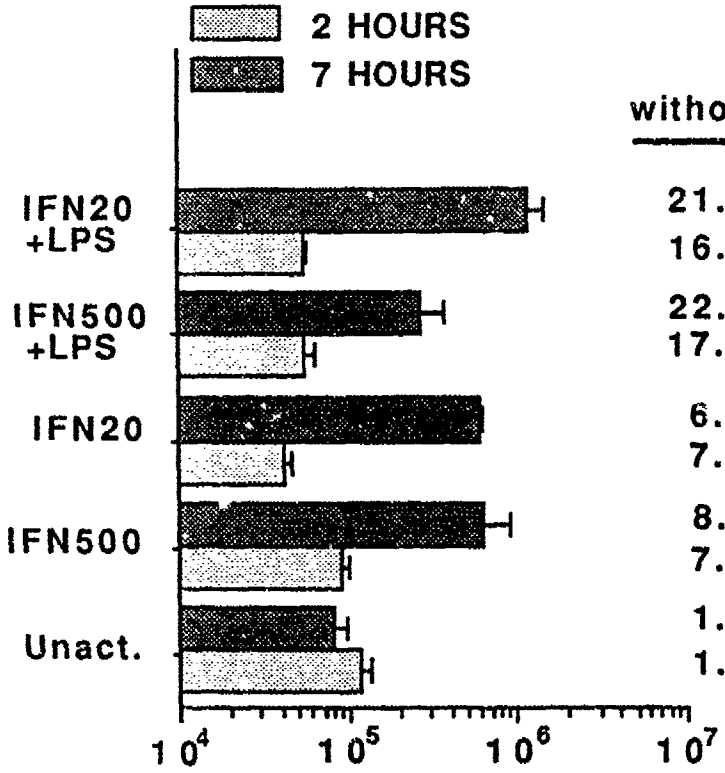
GROWTH OF *LISTERIA MONOCYTOGENES* IN Balb/c MACROPHAGES

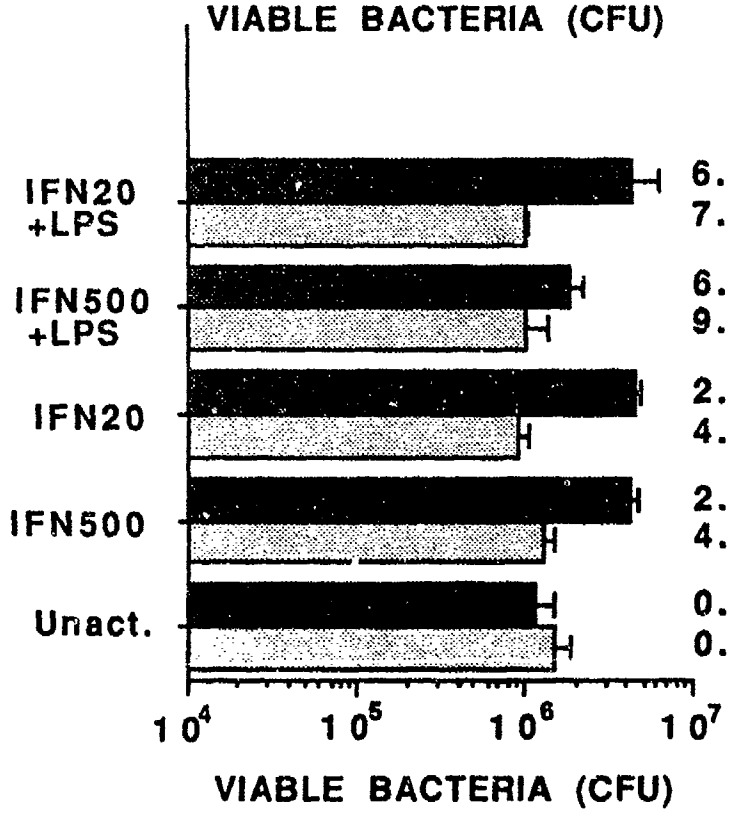


nitrate, and the cytostatic activity of macrophages toward tumor cells, yeasts, and protozoa (1, 2, 3). The role of these nitrogen oxides in bacteriostatic or bacteriocidal activities of macrophages has not been examined. The results shown in Figures 9-11 indicate that the presence or absence of L-arginine (the only known precursor of nitrogen oxides)(2) can affect the killing of *Listeria monocytogenes*. However, this is dependent on the mouse strain and the elicitation and activation stimuli and cannot be completely explained on the basis of the concentration of nitrite in the cultures. Resident macrophages from CD-1 mice (a strain which is moderately resistant to lethal infection with *Listeria monocytogenes*)(25) contained greater than 10-fold more viable *Listeria* when cultured in the absence of L-arginine than when cultured in the presence of L-arginine (Figure 9). This was true for unactivated macrophages and for macrophages activated with IFN or IFN plus LPS. Interestingly, these activation stimuli caused a loss of bacteriocidal activity (Figure 9). Similar results have been reported with proteose peptone-elicited macrophages from C57Bl/6 x DBA/2 F1 mice (15). The failure of activated CD-1 macrophages to kill *Listeria* was not due to a failure of the activating agents to induce nitrite production. Nitrite production was dramatically increased by all stimuli. Because the cultures were washed prior to the addition of bacteria, the nitrite concentrations noted in Figure 9 reflect only nitrites produced during the 2 or 7 hours after addition of bacteria. The concentrations of nitrites in cultures which were used to assess anti-bacterial activity were the same or greater than those in replicate cultures without bacteria. This demonstrates that the addition of *Listeria* did not inhibit the production of nitrites. Excluding L-arginine from the culture medium decreased nitrite concentrations in all cases, and there was a concomitant increase (greater than 10-fold in most cases) in viable *Listeria* at 2 hours and 7 hours. However, there are indications that the increase in bacterial numbers may not be entirely due to decreases in nitrite production. For example, the nitrite concentration at 2 hours in the arginine-free cultures treated with IFN at 500U/ml and LPS was 8.40±0.66 μM. A similar concentration (9.22±0.14 μM) was noted at 2 hours in the arginine-containing culture with IFN at 20U/ml, yet the concentration of *Listeria* in the former culture was approximately 20-fold greater than in the latter. The absence of arginine in macrophage cultures for 7 hours did not result in the death or generalized dysfunction of the cells. This is indicated by the unaltered bacteriocidal activity of some cultures in subsequent experiments in the absence of arginine (Figures 10 and 11). These

FIGURES 9 and 10. Effect of arginine on killing of *Listeria monocytogenes* and production of nitrite by resident or proteose peptone-elicited macrophages from CD-1 mice. Macrophages were activated for 18 hours and all cultures were washed to remove nitrites which had accumulated during the activation period. *Listeria monocytogenes* was added and two hours later, samples of supernatant fluid were obtained and assayed for nitrite, and the macrophages were washed thoroughly and lysed with distilled water to release *Listeria* for enumeration by the spread plate method. Nitrites in replicate macrophage cultures to which bacteria had not been added were also assayed. This process was repeated 7 hours after the addition of *Listeria* using another set of cultures. Values shown are means±standard deviation for triplicate cultures.

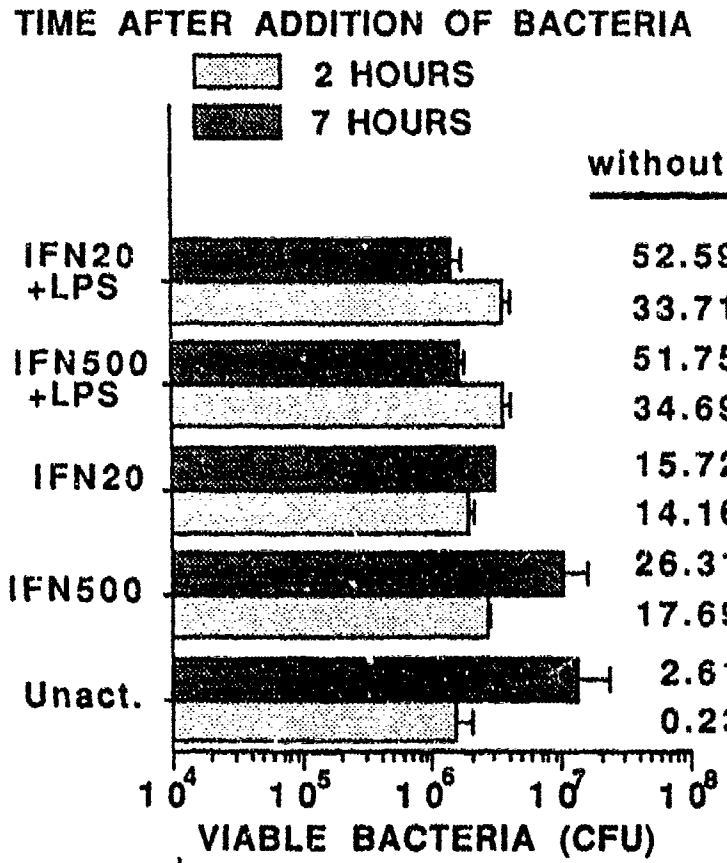
EFFECT OF ARGININE ON KILLING OF *LISTERIA MONOCYTOGENES* AND PRODUCTION OF NITRITE BY RESIDENT MACROPHAGES FROM CD-1 MICE

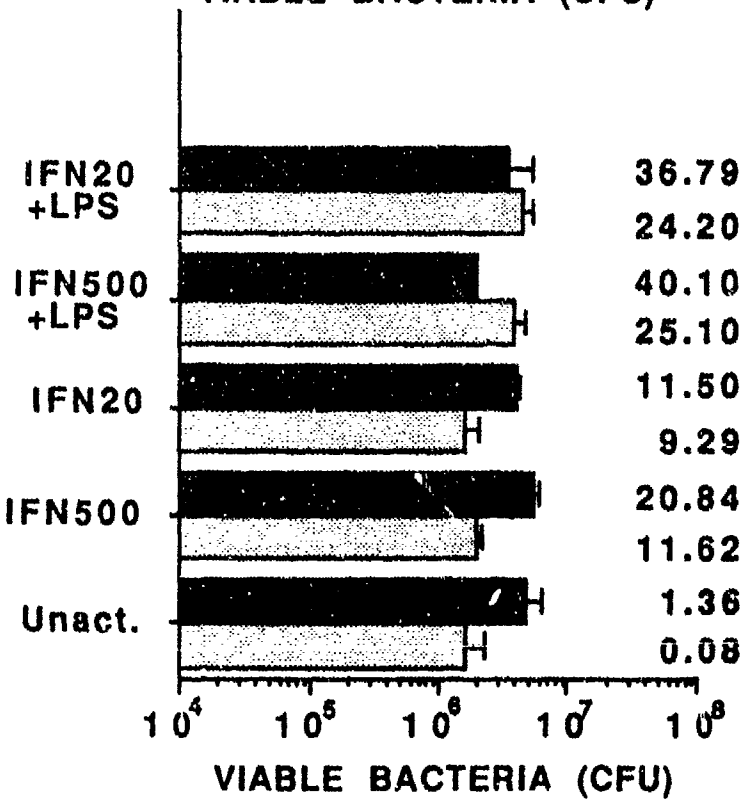




	NO_2^- (μM) without bacteria	NO_2^- (μM) with bacteria
Macrophages cultured with L-arginine		
IFN20 +LPS	21.32±1.98	23.11±0.80
	16.08±1.81	12.19±0.60
IFN500 +LPS	22.54±0.73	24.58±0.09
	17.97±1.34	13.20±0.23
IFN20	6.19±0.05	14.29±0.51
	7.21±0.46	9.11±0.14
IFN500	8.02±0.74	17.12±0.10
	7.70±0.23	10.00±0.19
Unact.	1.72±0.19	12.95±0.62
	1.40±0.42	3.39±0.33
Macrophages cultured without L-arginine		
IFN20 +LPS	6.65±0.26	7.14±0.37
	7.79±0.32	7.49±0.73
IFN500 +LPS	6.90±0.43	8.78±0.42
	9.44±0.19	8.40±0.66
IFN20	2.39±0.24	5.86±0.37
	4.34±0.51	5.35±0.45
IFN500	2.63±0.14	6.32±0.24
	4.46±0.73	5.65±0.16
Unact.	0.56±0.24	3.64±0.05
	0.00±0.00	2.29±0.38

EFFECT OF ARGININE ON KILLING OF *LISTERIA MONOCYTOGENES* AND PRODUCTION OF NITRITE BY PROTEOSE PEPTONE-ELICITED MACROPHAGES FROM CD-1 MICE





	NO_2^- (μM) without bacteria	NO_2^- (μM) with bacteria	
IFN20 +LPS	52.59±4.37	23.57±0.39	Macrophages cultured with L-arginine
	33.71±3.15	N.D.	
IFN500 +LPS	51.75±2.75	20.23±0.73	
	34.69±2.55	N.D.	
IFN20	15.72±0.16	13.17±1.26	
	14.16±1.17	N.D.	
IFN500	26.31±4.34	16.51±0.23	
	17.69±2.02	N.D.	
Unact.	2.61±0.33	10.89±1.84	
	0.23±0.23	N.D.	
IFN20 +LPS	36.79±0.64	10.93±1.45	Macrophages cultured without L-arginine
	24.20±2.51	N.D.	
IFN500 +LPS	40.10±6.29	10.66±0.26	
	25.10±0.72	N.D.	
IFN20	11.50±0.33	6.03±0.82	
	9.29±1.32	N.D.	
IFN500	20.84±2.57	8.84±0.73	
	11.62±1.72	N.D.	
Unact.	1.36±0.48	0.15±0.17	
	0.08±0.14	N.D.	

results point to the possible existence of an arginine-dependent anti-bacterial mechanism in addition to the production of nitrogen oxides.

The results shown in Figures 10 and 11 indicate that nitrogen oxides are not an important bacteriocidal mechanism in proteose peptone-elicited macrophages from CD-1 mice and are only important in a few cases in resident macrophages from DBA/2 mice. Bacterial concentrations were similar in arginine-free and arginine-containing cultures except in DBA/2 macrophage cultures treated with IFN alone, in which the number of bacteria was substantially greater in arginine-free cultures. These results suggest that the relative role of nitrogen oxides (and/or other arginine-dependent mechanisms) and other anti-microbial mechanisms depends on mouse strain and macrophage activation status.

Figure 12 is a composite of all assays of anti-*Listeria* activity showing the difference between the number of intracellular bacteria at 2 hours and at 7 hours. As noted above, this eliminates differences in bacterial numbers which may be due to different rates of phagocytosis and indicates only the rate of bacterial growth or death. Interstrain differences in the handling of *Listeria monocytogenes* are apparent. Unactivated resident macrophages from CD-1 mice and unactivated proteose peptone-elicited macrophages from C3H/HeN and DBA/2 mice are able to kill the bacteria. Unactivated proteose peptone-elicited macrophages from Balb/c mice and CD-1 mice and unactivated resident macrophages from DBA/2 mice did not prevent growth of intracellular *Listeria*. The effects of activating stimuli were also strain dependent. In most cases, full activation with LK SUP plus LPS or IFN plus LPS improved antimicrobial function. However, none of the activation stimuli affected the growth of *Listeria* in Balb/c macrophages, and all stimuli decreased the anti-microbial effectiveness of resident CD-1 macrophages. These strain differences as well as differences in elicitation and differences in the use of LPS could explain conflicting reports of enhancement (8), suppression (15), or no effect (26) of IFN on macrophage killing of *Listeria monocytogenes* and *Salmonella typhimurium*.

3. Additional Experiments (Not In The Original Proposal)

The results of this study and several published reports indicate that the production of nitrogen oxides by phagocytic cells is an inducible metabolic activity with several possible biological functions (1-5). Production of large quantities of nitrites by

FIGURE 11. Killing of *Listeria monocytogenes* by resident macrophages from DBA/2 mice cultured with or without arginine. Activation and anti-*Listeria* assays were performed as described in Figures 9 and 10. Values shown are means±standard deviation for triplicate cultures.

KILLING OF *LISTERIA MONOCYTOGENES* BY RESIDENT MACROPHAGES FROM DBA/2 MICE CULTURED WITH OR WITHOUT L-ARGININE

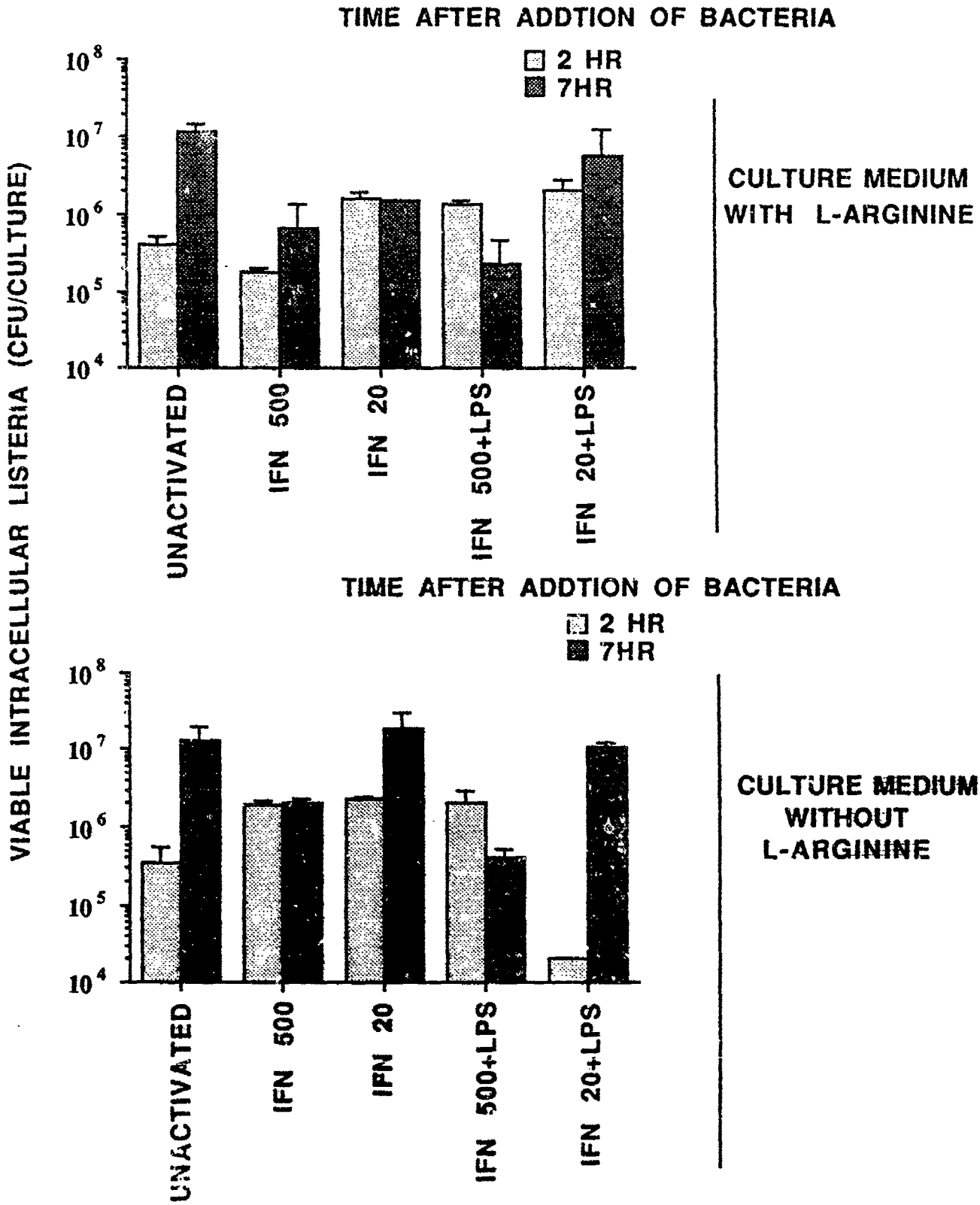
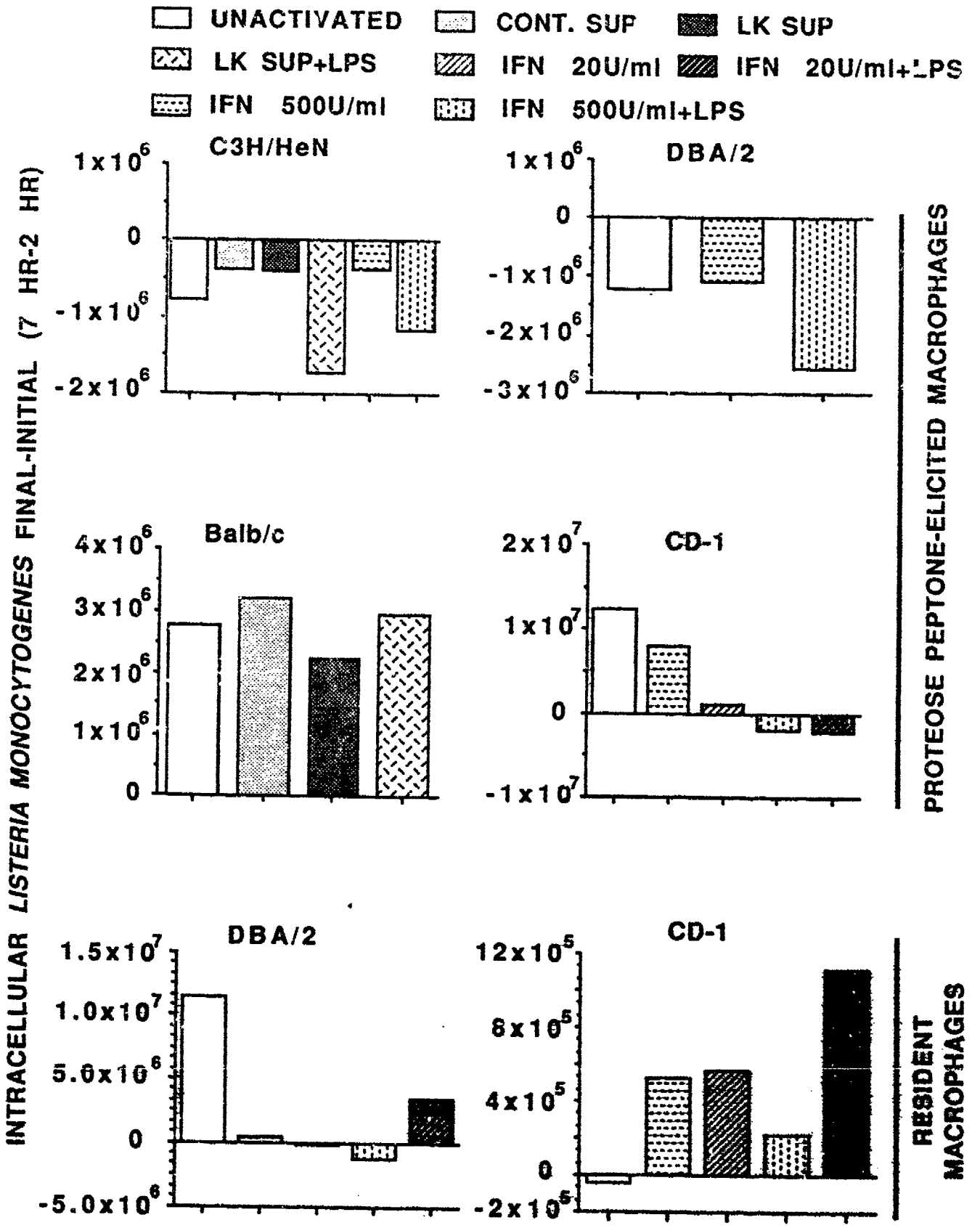


FIGURE 12. Changes in numbers of intracellular *Listeria monocytogenes* from 2 to 7 hours after addition of bacteria (CFU at 7 hours-CFU at 2 hours). This value represents the rate of killing of bacteria which have been ingested. Positive numbers indicate growth of bacteria within the macrophages, and negative numbers indicate a decrease in the number of intracellular bacteria. The data shown are from the experiments shown in the following figures: C3H/HeN, Figure 4; DBA/2 (proteose peptone-elicited), Figure 5; Balb/c, Figure 8; CD-1 (proteose peptone-elicited), Figure 10; DBA/2 (resident), Figure 11; CD-1 (resident), Figure 9.

CHANGES IN NUMBERS OF INTRACELLULAR *LISTERIA MONOCYTOGENES* FROM 2 TO 7 HOURS AFTER ADDITION OF BACTERIA (CFU AT 7 HOURS-CFU AT 2 HOURS)



inflammatory neutrophils isolated from rats has recently been reported (5), and it has been suggested that nitrogen oxides produced by neutrophils may be important mediators of anti-microbial action and tissue damage in acute inflammation (5). Whether this might also be the case in humans is not known. Although nitric oxide production by human neutrophils has been reported (27), the compound was detected by its very potent action as an inhibitor of platelet aggregation, and the quantities of nitrogen oxides produced were quite low. The following experiments were done to assess the capacity of human neutrophils to produce nitrogen oxides as indicated by the accumulation of a stable nitrogen oxide, nitrite.

Human neutrophils were isolated from blood samples provided by several volunteers by centrifugation over a discontinuous ficoil gradient (1.077g/ml over 1.119g/ml). Neutrophils were 96-98% pure (by differential staining), and greater than 95% viable (by trypan blue exclusion). To monitor the viability and metabolic activity of neutrophils during culture, an assay was developed which is based on the ability of viable cells to reduce the tetrazolium compound MTT to an insoluble formazan which can be quantitated spectrophotometrically. We found that MTT was also an excellent indicator of neutrophil respiratory burst activity. A manuscript describing this assay is in press (a copy is included in this report). This assay was used to detect a stimulus-dependent suppression of the respiratory burst by two organophosphorus compounds, profenofos and phenyl saligenin cyclic phosphate (see appended abstract by E.L. Padgett and S.B. Pruett). The latter compound is an analog of the active metabolite of tri-*o*-cresyl phosphate, a compound of interest to the Air Force.

In several experiments using neutrophils and autologous serum from 5 different donors nitrite production was undetectable at 1, 3, 8, or 24 hours of culture. The addition of Group B Streptococci (heat-killed) to trigger the respiratory burst produced measurable nitrites (less than 5μM) from 1 to 24 hours after triggering, but two other triggering agents (phorbol myristate acetate and opsonized zymosan) produced no detectable nitrites. The viability and respiratory burst activity in selected experiments was monitored by the MTT assay, and the cells were viable and metabolically active. Since the initial nitrogen oxidation product (nitric oxide) can react with superoxide produced during the respiratory burst, superoxide dismutase (SOD) was included in some experiments to eliminate superoxide. As expected, SOD inhibited reduction of MTT (see attached manuscript), but no increase in nitrite concentration was observed. The possibility remained that neutrophils activated by

inflammatory stimuli, but not triggered for the respiratory burst, were the best producers of nitrogen oxides. This was examined by treating neutrophils with the priming agents LPS (28), Interferon-gamma (29), or a combination, and monitoring nitrite concentrations at selected time points from 1-24 hours. Nitrites were not detectable in culture supernates at any time. The cell density for these studies was $3x10^5$/well (0.2ml). The same density of murine macrophages produced up to 50μM nitrites in other experiments in this study (Figure 10)

These results suggest that human neutrophils are not capable of producing the substantial quantities of nitrogen oxides which are required for anti-microbial (3) and, presumably, for tissue damaging effects. Although unlikely, it remains possible that neutrophils activated by inflammatory processes *in vivo* may acquire this capability, and this should be the subject of future research.

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c. Publications

1. Pruett, S.B., and Loftis, A. (1990) Characteristics of MTT as an indicator of viability and respiratory burst activity of human neutrophils. *Int. Arch. Allergy Appl. Immunol.* In press. (a copy of the galley proof is appended).
2. Higginbotham, J.N., and Pruett, S.B. (1990) Thiol production as an indicator of exposure of murine macrophages to activating stimuli. In preparation.
3. Higginbotham, J.N., and Pruett, S.B. (1990) Interstrain differences in the induction of Listericidal activity in murine macrophages by Interferon-gamma. In preparation.
4. Padgett, E.L., and Pruett, S.B. (1990) Human neutrophils triggered or primed *in vitro* are unable to produce nitrites from L-arginine. In preparation.

d. Professional personnel associated with the research effort

Two Master's degree students, Jimmy N. Higginbotham and Eric L. Padgett were involved in this study. Both of them plan to graduate in 1991.

e. Interactions

i) Posters presented at the Annual Meeting of the Society of Toxicology, Miami, February, 1990 (Copies of abstracts are appended).

Padgett, E.L., and Pruett, S.B., (1990) Effect of organophoshporus compounds on production of H_2O_2 by human neutrophils. *Toxicologist* 10:291.

Higginbotham, J.N., and Pruett, S.B. (1990) Relationship of selected functions and activation status. *Toxicologist* 10:292.

ii) Consultative and advisory functions

During this project, I have maintained a collaborative research effort with Dr. Johnathan Kiel, USAF School of Aerospace Medicine, Brooks AFB, TX. This work has resulted in the preparation of a manuscript, "Stress Response of Macrophages to Hyperthermia and Lipopolysaccharide Measured by Extracellular Thiol Production and Cell Survival" which will soon be submitted for publication. Dr. Kiel and I plan to continue this collaborative effort.

f. Discoveries, Inventions, Specific Applications

Assessment of macrophage function and its alteration by physical or chemical agents has proven to be one of the most technically difficult areas in immunotoxicology. The results of this study and collaborative efforts with Dr. J.L. Kiel indicate that thiol production by macrophages in culture could be an excellent indicator of the activation status of these cells. This should allow standardization of the macrophage populations used in immunotoxicological assessments.

A new assay for viability and respiratory burst activity of human neutrophils was developed. This assay can be used to assess the effects of physical or chemical agents on these cells (see appended abstract by E.L. Padgett and S.B. Pruett).

1166 **RELATIONSHIP OF SELECTED MACROPHAGE FUNCTIONS AND ACTIVATION STATUS.** J N Higginbotham and S B Pruett, Dept. Biol. Sci., Miss. State U., Miss. State, MS. Sponsor: J E Chambers

Several toxicants affect one or more of the immunologically important functions of macrophages. Simultaneous modulation of several of these functions also occurs in response to various macrophage activation stimuli. We report here the simultaneous assessment of several relevant functions of macrophages in different states of activation. Macrophages were obtained from the peritoneal cavities of DBA/2 mice 3 days after elicitation with proteose peptone. The cells were pooled and placed in triplicate microtiter cultures and treated with recombinant mouse interferon gamma (500U/ml) (IFN), IFN and lipopolysaccharide (10ng/ml) (LPS), or culture medium only. Production of thiols in the medium was increased by IFN and further increased by IFN/LPS. Production of H_2O_2 after stimulation with phorbol myristate acetate was increased equally by IFN and IFN/LPS. Tumor cytotoxicity was only evident in macrophages treated with IFN/LPS, and listericidal activity was decreased by IFN and further decreased by IFN/LPS. These results provide a point of reference for future studies of the effects of immunotoxicants on selected macrophage functions. Supported by AFOSR 89-0361.

1162 **EFFECT OF ORGANOPHOSPHORUS COMPOUNDS ON PRODUCTION OF H_2O_2 BY HUMAN NEUTROPHILS.** E L Padgett and S B Pruett, Dept. Biol. Sci., Miss. State U., Miss. State, MS Sponsor: J E Chambers.

It has recently been suggested that neutrophil proteases play an important role in the events leading to H_2O_2 production. Because many organophosphorus compounds (OPs) irreversibly inhibit serine proteases, we examined the effects of several OPs on production of H_2O_2 by human neutrophils. H_2O_2 production was measured by oxidation of phenol red in the presence of horseradish peroxidase and was triggered by phorbol myristate acetate (PMA)(200nM) or opsonized zymosan (.1-1mg/ml). Several compounds were tested initially at 100μM. Diazinon, chlorpyrifos, chlorpyrifos oxon, aspon, methyl parathion, and EPN-oxon had no significant effect. Profenofos and phenyl saligenin cyclic phosphate inhibited PMA-induced H_2O_2 production by approximately 50% but did not inhibit opsonized zymosan-induced H_2O_2 production. The results indicate that production of H_2O_2 can be inhibited by concentrations of OPs which could be attained *in vivo* without lethal neurotoxicity. Supported by AFOSR 89-0361.

Characteristics of MTT as an Indicator of Viability and Respiratory Burst Activity of Human Neutrophils[1]

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Abstract. In the present study, we have examined the suitability of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) for assessing the viability and respiratory burst activity of human neutrophils. MTT is a good indicator of the relative viability of neutrophils which have been in culture up to 24 h, if sufficient serum (10% fetal calf serum) is present in the medium and the cells being compared have been in culture for a similar period of time. However, it is not suitable for exact assessment of percent viable cells in a cell population with low viability. The effectiveness of MTT as an indicator of respiratory burst activity is demonstrated by the sharp increase in MTT reduction induced by respiratory burst stimuli and by the ability of superoxide dismutase to inhibit 75% of MTT reduction by stimulated neutrophils. Unlike nitroblue tetrazolium (which is reduced primarily intracellularly) and cytochrome c (which is reduced extracellularly), MTT is apparently reduced both intracellulary and extracellulary by activated neutrophils.

Assessment of neutrophil viability and respiratory burst activity are often important in studies of the damaging effects of inflammatory neutrophils (PMN). However, conventional viability assays may be unreliable due to adherence of activated neutrophils to surfaces and due to fragmentation of dead cells. An in situ assay for viability would solve these problems. Ideally, respiratory burst activity should also be measurable with a simple assay using the same culture conditions. In this report we have investigated the use of the tetrazolium compound 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to assay both parameters. Viable cells reduce MTT to a formazan which is soluble in aqueous isopropanol solution and exhibits an absorbance maximum at approximately 590 nm [1]. This phenomenon is the basis for a widely used colorimetric cell growth and viability assay [1–4]. Reduction of tetrazolium compounds is also used to measure the respiratory burst of phagocytic cells. The most widely used compound for this purpose is nitroblue tetrazolium (NBT). This compound is reduced by superoxide which is produced during the respiratory burst [5] and possibly also by intracellular diaphorases [6]. Essentially all the formazan produced from NBT by phagocytic cells is intracellular or closely associated with cells [6].

The chemical similarities between NBT and MTT and an observation by M. Salin and co-workers [pers. commun.] that MTT is reduced by PMN prompted us to investigate the use of MTT to assay both the viability and the respiratory burst activity of PMN.

All reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.), and stock solutions were stored in small volumes at −80 °C until needed. Blood for isolation of PMN was obtained by venipuncture into tubes with EDTA as anticoagulant. The 5 donors (2 female and 3 male) were healthy volunteers aged 20–35 years. There were quantitative differences in MTT reduction by PMN from different donors, but patterns of survival and response to stimuli were similar for cells from all donors. PMN were isolated using a discontinuous Ficoll gradient consisting of 9 ml of 1.077 g/ml Ficoll (Histopaque; Sigma Chemical Co.) layered over 9 ml of 1.119 g/ml Ficoll in a 50-ml conical centrifuge tube. Eighteen milliliters of blood was layered onto the gradient and the tube was centri-

[1] Supported by grant AFOSR-89-0361 from the US Air Force Office of Scientific Research.

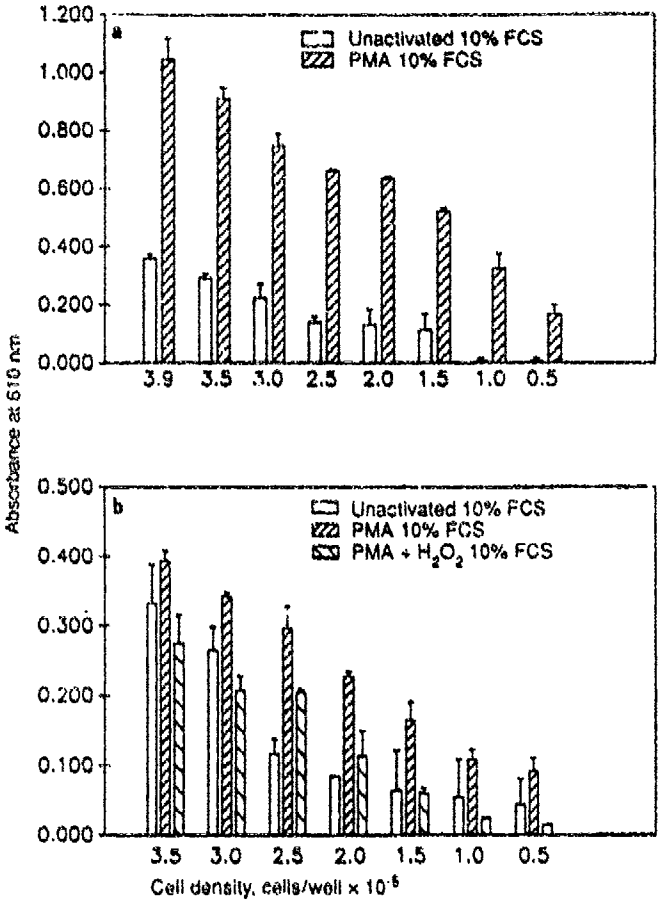

Fig. 1. Effect of increased serum concentration on the ability of neutrophils to reduce MTT after 24 h in culture. Neutrophils were cultured for 2 h (a) for 24 h (b) in medium with 10% FCS before addition of MTT. Some cultures were stimulated with PMA (200 n*M*) immediately after addition of MTT. Absorbance at 610 nm was measured 2 h after MTT addition. H_2O_2 (1 m*M*) was added to some wells at the beginning of the culture to assess the effect of decreased cell viability on MTT production by stimulated neutrophils. The mean ± SD is shown for duplicate wells of all treatments.

fuged at 600 *g* for 30 min. PMN isolated from the lower interface were 96–98% pure as indicated by differential counts of stained (Camco Quick Stain II; Baxter Scientific) smears. The isolated PMN were washed twice with calcium and magnesium-free PBS containing 0.1% bovine serum albumin (low endotoxin BSA; Sigma Chemical Co.). All buffers and media were adjusted to pH 6.9–7.0 immediately before use. The cells were counted by hemocytometer and resuspended in assay medium which consisted of RPMI 1640 with penicillin-streptomycin supplement with fetal calf serum (FCS) at 1, 2, or 10%. The MTT assay was performed by two different methods. In the experiment shown in figure 2, the method of Denizot and Lang [1] was used. Briefly, 10 µl of MTT solution was added to 100 µl of cell suspension in a 96-well plate. After incubation, the supernate was inverted to remove supernates and the formazan crystals which adhered to the plate were resuspended in isopropanol (200 µl) and absorbance at 610 nm was determined using a plate reader (Multiskan; Flow Labs). Because of occasionally disparate results among replicates using this method, all other experiments employed the method of Mosmann [2] in which acidified isopropanol is added to the wells without first removing the culture medium. Due to the rapid response to some of the stimuli used, MTT was added to all wells before stimuli were added. Preliminary experiments indicated that absorbance at 610 nm reached a maximum 2 h after addition of stimulus. Therefore, absorbance was measured 2 h after addition of MTT (and stimulus) in all experiments. In some experiments, cell viability was assessed by trypan blue exclusion. If the total number of cells (live plus dead) at the end of the culture period was less than the initial cell number, the difference was assumed to be due to fragmentation of dead cells, and the 'missing' cells were regarded as dead when percent viability was calculated [7].

PMN viability was examined at several time points in a 24-hour culture by trypan blue exclusion and MTT reduction. The ability of unstimulated PMN (2×10^5 cells/0.2 ml) cultured in 1 or 2% FCS to reduce MTT decreased to nearly undetectable levels by 24 h in culture (results not shown). Trypan blue exclusion indicated that viability also decreased, but 42% of the cells were still viable at 24 h. Opsonized zymosan (OpZy) (0.2 mg/ml) [8] and formyl methionine leucine phenylalanine (FMLP) (10^{-6} *M*) increased MTT reduction during the first 2 h of culture (absorbance at 610 nm for controls = 0.176 ± 0.021; OpZy = 0.368 ± 0.041; FMLP = 0.221 ± 0.015). However, these agents had little effect on MTT reduction or viability at 5, 10, or 24 h. In contrast, phorbol myristate acetate (PMA) caused an initial increase in MTT reduction followed by a decrease to background levels within 4 h. This paralleled a loss of cellular integrity as observed microscopically, but accurate data could not be obtained for trypan blue exclusion due to the adherence of treated cells to the wells.

Increasing the serum concentration from 1 or 2%

to 10% caused unstimulated PMN to retain most of their MTT reductive capacity during a 24-hour culture (fig. 1). However, MTT reduction in response to PMA decreased by more than 50% as indicated by comparing results in the 2-hour culture with those in the 24-hour culture (fig. 1). Cell death induced by exogenous H_2O_2 decreased the quantity of MTT reduction noted in cells stimulated with PMA after 24 h in culture (fig. 1). The decreases in MTT reduction paralleled the diminished numbers of intact cells observed microscopically and in micrographs (not shown).

The simplest approach in using the MTT assay to determine the percent viability of an unknown cell population would be to establish a standard curve of cell number vs. MTT reduction (absorbance at 610) using a population of cells with known, high viability. Given the initial cell number in an unknown sample, the number of viable cells remaining at a given time point (and thus, the percent viability) could be determined by comparison to the standard curve. However, such a procedure would only be valid if the standard curve and a similar curve obtained using a partially viable cell population have similar slopes. The results shown in figure 2 demonstrate that this is not the case for the predominantly viable and predominantly nonviable cell populations used in this experiment. The slopes are significantly different ($p < 0.05$; determined as described by Zar [9]) for these two populations, suggesting that it would not be valid to use a standard plot with live cells to determine percent viability of an unknown sample. Thus, MTT can be a useful indicator of the relative viabilities of PMN populations cultured under similar conditions, but it may not be suitable for precise quantitation of percent viability in predominantly nonviable cultures.

Superoxide dismutase (SOD) at 250 U/ml inhibited MTT reduction by PMA-stimulated PMN by approximately 50%, but inhibition was minimal in unstimulated cells or with boiled SOD (results not shown). Higher concentrations of SOD diminished MTT reduction by unstimulated cells but, once again, not to as great an extent as noted for PMA-stimulated cells. The absorbance value for non-activated cells (3.5×10^5 cells/well) was 0.176 ± 0.015, whereas the value in the presence of 1,250 U/ml SOD was 0.106 ± 0.005. For PMA-activated cells (1.5×10^5 cells/well), the corresponding values were 0.182 ± 0.035 and 0.044 ± 0.010, respectively. Thus, MTT reduction by activated PMN is predominantly superoxide-mediated. In addition, the data in figure 1 indicate that at low cell densities, only stimulated PMN reduce measurable quantities of MTT. Under these conditions, this assay would exclusively detect MTT reduced as a result of the respiratory burst. Micrographs taken 30 min after PMA stimulation demonstrate abundant, extracellular formazan crystals as well as cell-associated formazan (fig. 3). SOD (625 U/ml) inhibited formation of most of the extracellular formazan, but deposits which appeared to be intracellular were still present (fig. 3). This reduction of MTT both intracellularly and extracellularly contrasts with the exclusively extracellular reduction of cytochrome c [6] and the exclusively intracellular reduction of NBT [6].

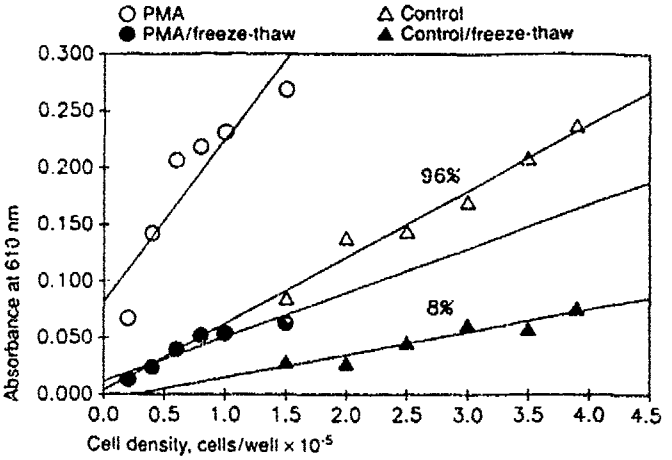

Fig. 2. Comparison of MTT reductions vs. cell density plots for predominantly live and predominantly dead (freeze-thaw) neutrophils. Neutrophils at 2×10^6/ml in medium with 10% FCS were incubated at room temperature for 20 min or placed in a −80°C freezer for 15 min then thawed in a 37°C water bath (freeze-thaw) before being placed in culture wells at the indicated densities. MTT was added to all wells and PMA (200 n*M*) to some, and absorbance at 610 nm was measured 2 h later. Viability of unstimulated cells was determined by trypan blue exclusion at the same time (2 h after MTT addition) and is shown above the corresponding plots. Values shown are means for duplicate wells. Standard deviations were never greater than 20% of the mean.

In summary, MTT can be used to assess the relative viability and the respiratory burst activity of PMN, but may not be suitable for precise quantitation of percent viability of predominantly nonviable cell populations. It appears to be reduced both intracellularly and extracellularly by activated PMN, and is, therefore, unique among the agents most commonly used to assess the respiratory burst.

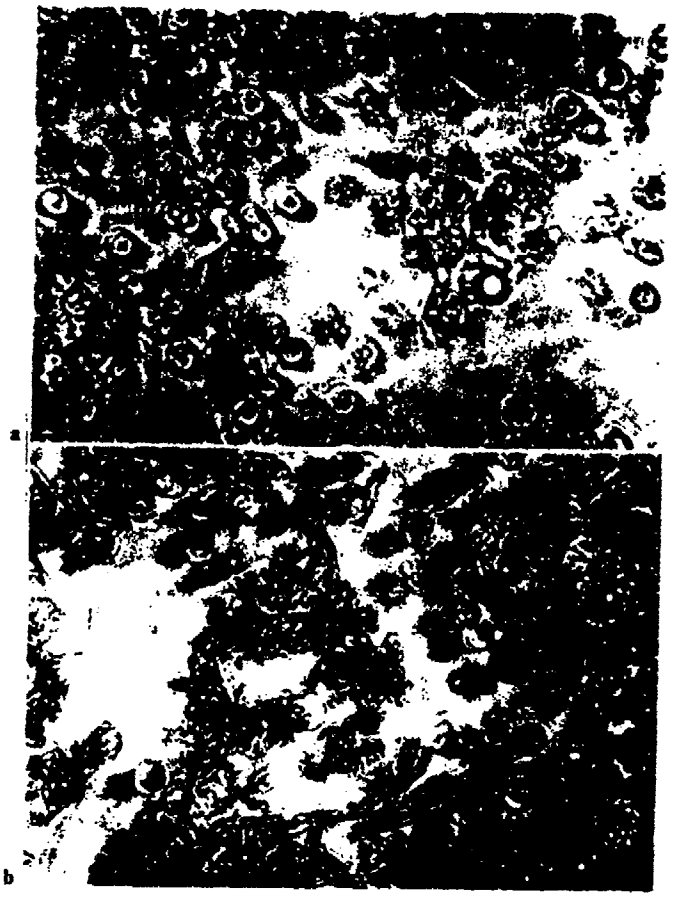

Fig. 3. Effect of SOD on generation of extracellular formazan crystals by neutrophils. Neutrophils (2×10^5/well) were treated with MTT and the cells in **a** received SOD (625 U/ml) and PMA (200 n*M*), whereas cells in **b** received PMA (200 n*M*) but not SOD. Magnification is 400× and the micrographs were taken 30 min after addition of MTT.

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Received: March 5, 1990
Accepted after revision: May 21, 1990

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